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14. ABSTRACT
The primary goal of this collaborative research project is to develop next generation engineered nerve guide conduits (NGCs) with aligned nanofibers and favorable release kinetics of neurotrophic factors to help improve surgical outcomes after injuries involving peripheral nerves.
In the fourth year of the work, we completed the development of the NGCs and tested various combinations of NGCs in a rat sciatic nerve regeneration model. After obtaining the IACUC and ACURO approval for the large animal validation study, we have completed most of the groups. Currently last 2 groups are being monitored and will be analyzed in the last year of no-cost extension (5th year).

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Introduction:
Peripheral nerve injury is a common complication of complex tissue trauma and often results in significant disability in war injuries. Regeneration of peripheral nerves is often incomplete and in complex war injuries donor nerves are difficult to find for nerve repair. Nerve guide conduits (NGCs) made of biodegradable materials offer a potential solution to this problem. Based on our previous accomplishments in developing a nanofiber containing NGCs, the primary goal of this collaborative research project is to develop new nanofiber NGCs with improved nanofiber guidance cue and modulated trophic factor delivery capabilities that promise faster nerve regeneration and better functional recovery.

Body:

Statement of Work (Proposed Tasks)

The goals of Year 2 included optimization of nanofiber nerve guide design to provide i) contact guidance cue (nanofiber diameter, degradation rate, and fiber density/distribution) and ii) modulated neurotrophic factor delivery (factor choice, concentration range and gradient configuration) for regenerating axons and Schwann cells, and assessing the effects of each modification in a rat model of nerve regeneration. Parts of these goals were not completed in Year 2 but were completed in Year 3. Furthermore, in Year 3 we had planned to carry out Task 3, large animal validation study, but this was delayed into the 4th year (a no-cost extension year) because of the delays in manufacturing and testing the optimum configuration of the NGCs. We recently obtained another year of no-cost extension as the dog studies had to be staggered and completion extended beyond the 4th year.

Goals for Year 2 and 3 were outlined in the original Statement of Work as:

Task 2: To assess nerve regeneration rate and functional recovery in a rat model of nerve repair, and to optimize nerve guide configurations

- 2a. To manufacture of nanofiber nerve guides of without neurotrophic factor loading
- 2b. To evaluate the effect of nanofiber diameter and degradation rate on nerve regeneration in the rat sciatic model
- 2c. To manufacture nanofiber nerve guides with optimum neurotrophic factor loading
- 2d. To evaluate the effect of different neurotrophic factors on nerve regeneration in the rat sciatic model
- 2e. To prepare and obtain regulatory approval for the dog studies

Task 3: To demonstrate efficacy in a large animal model using optimized nerve guide from Aim 2, as a prerequisite for clinical translation

- 3a. To manufacture the nanofiber nerve guides to be used in the dog peroneal nerve repair model (months 23-24)
- 3b. Surgical repair of the peroneal nerve using the nanofiber nerve guides; total 60 dogs used (months 24-25)
- 3c. Longitudinal gait evaluation for foot drop (months 24-33)
- 3d. Retrograde labeling and tissue harvesting (months 32-33)
- 3e. Nerve morphometry (months 33-35)
- 3f. Histopathological evaluations (months 33-35)

Progress

Beginning of Year 4 was spent on wrapping up the remaining few groups in rat studies to optimize the design of the conduits. However, most of the year was committed to Task 3. Since we could not house all dogs in all groups at the same time and carry out the surgeries and behavioral tests in a reliable manner, we staggered the surgeries and have completed all of the animals in groups 1, 2 and

3 including harvesting of the nerve biopsies. We have not done the nerve morphometrics because we want to wait until we have all of the groups so we can evaluate them in an unbiased blinded manner. Groups 4 and 5 surgeries have also been completed and the animals are being monitored until the tissue harvest takes place in few months.

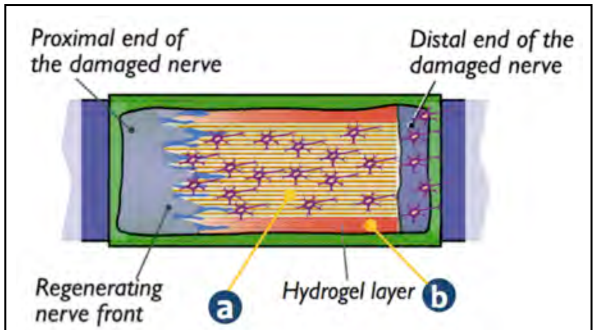

Fig. 1. Design of the nanofiber nerve guides incorporating the nanofiber guidance cues (a) and neurotrophic factor gradient loaded in the hydrogel layer (b) between the outer membrane and nanofibers.

Specific experiments were completed in preparation for the canine study. Enhancements to the *in vitro* migration analysis platform allowed for high throughput analysis of Schwann cell migration in response to fiber-mediated topographical guidance and neurotrophic factor gradient-mediated chemotrophic guidance. Improvements in our *in vitro* platform as well as improved design of the nerve conduit allowed for significant progress to be made on optimizing nerve conduits incorporating both nano/microfiber-mediated contact guidance and controlled release of neurotrophic factor gradients (Fig. 1).

In Year 4, before starting the dog studies, we completed Task 2 and finalized the design of the nerve conduits.

Optimizing Nanofiber Guidance (tasks 2a-2d)

Final Composition of the Optimized NGC

Based upon the results of the gradient tier of nerve guides, we determined the optimal design for the dog study. To verify the results, we have run one final set of in vivo experiments in October, 2013. We have implanted 4 more rats with Group 19 nerve guides ("S" design with steep gradient of GDNF) to have 8 total rats in the group to prove reproducibility. We have also implanted 8 rats with group 20 nerve guides, with the steep GDNF gradient, but without the "S" shape, with only a single, peripheral layer of aligned fibers, to determine the contributing effects of the S-shape versus gradient.

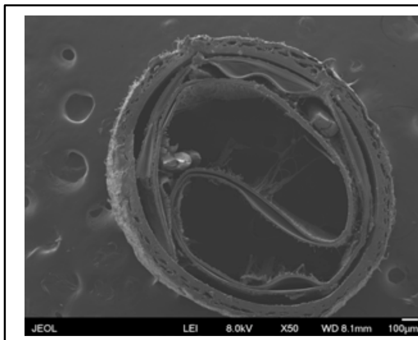

Figure 2: Cross section scanning electron micrograph of S-shaped configuration of nerve conduits

Evaluation of these final groups was completed in January and February of 2014. It appears that the optimum configuration of the nanofiber nerve guides is based on the data from Group 19 of the rodent studies. In this configuration, the nanofibers are distributed in an S-shaped configuration. This format allows the lumen to remain open after nerve repair by adding internal resistance to compression and increases the surface area of nanofibers for regenerating axons to attach and extend.

In this group, the other parameters that had been tested previously included the steepness of the gradient loading. Compared to the Group 18 (shallow gradient), there was better regeneration in Group 19 (steep gradient), so we decided on the steep gradient of GDNF in our nanofibers for the dog studies.

Another comparison that we carried out was the relevance of S-shaped nanofibers versus a single layer (Group 20) with steep gradient release of GDNF. As expected based on previous groups, the S-shaped group (Group 19) was superior to a single layer of nanofibers.

Large animal validation studies (Tasks 3a-3f)

Task 3: To demonstrate efficacy in a large animal model using optimized nerve guide from Aim 2, as a prerequisite for clinical translation
3a. To manufacture the nanofiber nerve guides to be used in the dog peroneal nerve repair model
3b. Surgical repair of the peroneal nerve using the nanofiber nerve guides
3c. Longitudinal gait evaluation for foot drop
3d. Retrograde labeling and tissue harvesting
3e. Nerve morphometry
3f. Histopathological evaluations

Task 3a is completed:
We've manufactured and transplanted into dogs all of the specific types of nerve conduits and control groups (see below for information about specific groups).

Task 3b is completed:
We have completed all of the surgeries:

- Group 1: Autograft
- Group 2: Neurogen collagen based hollow nerve conduit (currently FDA approved for short segment repairs)
- Group 3: Nanofiber nerve conduit (S-shaped) without any neurotrophic factor
- Group 4: Nanofiber nerve conduit (S-shaped) with uniform loading of neurotrophic factor, GDNF
- Group 5: Group 4: Nanofiber nerve conduit (S-shaped) with steep gradient loading of neurotrophic factor, GDNF

Task 3c is ongoing:
We have completed the walking track analysis in Groups 1, 2 and 3 and carrying it out in groups 4 and 5.

Task 3d (tissue harvesting) is ongoing:
We harvested tissues from Groups 1, 2 and 3. Groups 4 and 5 will be harvested in June and July 2015.

Task 3e (nerve morphometry) is ongoing
Distal deep peroneal nerves and conduits harvested from Groups 1, 2 and 3 have been processed by the electron microscopy laboratory for plastic embedding, sectioning and staining. We have not counted the number of regenerated axons, as we want to wait and have all 5 groups completed before counting them so we can mask the identity of the samples and count them in an unbiased blinded manner.

Task 3f (histopathological evaluation) is ongoing:
Parts of the conduits from Groups 1, and 3 have been fixed and immunostained for analysis of fibrosis, regeneration and tissue rejection. These again will be quantified in an unbiased blinded manner once we have all 5 groups ready.

Key Research Accomplishments:

Refinement of the nanofiber NGCs:

- Increased nanofiber surface area
- Fine-tuning of nanofiber degradation rate
- Gradient loading of neurotrophic factors
- Evaluation of Schwann cell invasion and motility on nanofiber NGCs

Validation studies in the rat sciatic nerve regeneration model

- Tested the role of increased nanofiber surface area (spiral design)
- Tested the role of gelatin hydrogel with GDNF loading
- Tested the role of nanofiber density
- Tested the role of gradient neurotrophic factor loading
- Tested the optimum combination of NGCs

Validation studies in the large animal peroneal nerve repair model

- Completed manufacture of all nerve guidance conduits for use in dog studies
- Completed the surgeries in all 5 groups
- Harvested the tissues in Groups 1, 2 and 3

Reportable Outcomes

Manuscripts Published

Krick K, Tammia M, Martin R, Höke A, Mao HQ. Signaling cue presentation and cell delivery to promote nerve regeneration. Current Opinions in Biotechnology, 22(5): 741-746 (2011).

(Not directly supported by this grant, but very much relevant to overall aims of enhancing peripheral nerve regeneration using topographical cues:
Ren YJ, Zhang S, Mi R, Liu Q, Zeng X, Rao M, Hoke A, Mao HQ. Enhanced differentiation of human neural crest stem cells towards the Schwann cell lineage by aligned electrospun fiber matrix. Acta Biomater. 2013 Aug;9(8):7727-36. PMID: 23628775

Jiang X, Mi R, Hoke A, Chew SY. Nanofibrous nerve conduit-enhanced peripheral nerve regeneration. J Tissue Eng Regen Med. 2012 Jun 15. PMID: 22700359

Zhang S, Liu X, Barreto-Ortiz SF, Yu Y, Ginn BP, DeSantis NA, Hutton DL, Grayson WL, Cui FZ, Korgel BA, Gerecht S, Mao HQ. Creating polymer hydrogel microfibres with internal alignment via electrical and mechanical stretching. Biomaterials. 2014 Mar;35(10):3243-51. Epub 2014 Jan 15. PMID: 24439410

Sarhane KA, Ibrahim Z, Cashman C, Martin R, Krick K, Tuffaha SH, Broyles JM, Pan B, Prasad N, Tehrani S, Alrakan M, Wallner C, Cooney DS, Mi R, Höke A, Lee WP, Mao HQ, Brandacher G. Enhanced nerve regeneration by minimizing intraneural scarring using a semi-permeable nanofiber wrap. Plast Reconstr Surg. 2014 Oct;134(4 Suppl 1):54-5. PMID: 25254753

Scientific Presentations

Krick KD, Khademhosseini A, Höke A* and Mao HQ*. Neurotrophic Factor Gradient Delivery for Migration Guidance of Schwann Cells, Poster Presentation at the BMES Annual Meeting, Seattle, September 2013

Sarhane KA, Tuffaha SH, Krick K, Cashman C, Budihardjo JD, Broyles JM, Martin R, Abraham J, Ibrahim Z, Mi R, Cooney DS, Hoke A, Lee WP, Mao HQ, Brandacher G. A Critical Analysis of Peripheral Nerve Regeneration in a Chronic Denervation Rat Model Using a Sustained Biomaterial-Based Delivery of GDNF, Chondroitinase, and GDNF+Chondroitinase Growth Factors. Plast Reconstr Surg. 2015 May;135(5S Suppl):87. PMID: 25915334

Yalanis GC, Reddy S, Martin R, Choi J, Brandacher G, Mao HQ, Sacks JM. An Electrospun Fiber-Hydrogel Composite with Interfacial Bonding for Soft Tissue Regeneration in vivo. Plast Reconstr Surg. 2015 May;135(5S Suppl):90-91. PMID: 25915340

Conclusion:
We have evaluated the role of multiple variables relevant to design and optimization of the nanofiber NGCs and tested them in the rat studies. We have obtained IACUC and ACURO approval for the large animal validation study and have completed majority of those experiments. We plan to complete remaining the large animal validation studies in this no-cost extension year and report our final outcomes at the end of the year.

References:
None

Appendices:
None